INFLUENCING THE ATTACHMENT OF BACTERIA THROUGH LASER SURFACE ENGINEERING

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Abstract

Bacteria have evolved to become proficient at adapting to both extracellular and environmental conditions, which has made it possible for them to attach and subsequently form biofilms on varying surfaces. This has resulted in major health concerns and economic burden in both hospital and industrial environments. Surfaces which prevent this bacterial fouling through their physical structure represent a key area of research for the development of antibacterial surfaces for many different environments. Laser surface treatment provides a potential candidate for the production of anti-biofouling surfaces for wide ranging surface applications within healthcare and industrial disciplines. In the present study, a KrF 248 nm Excimer laser was utilized to surface pattern Polyethylene terephthalate (PET). The surface topography and roughness were determined with the use of a Micromeasure 2, 3D profiler. Escherichia coli (E. coli) growth was analysed at high shear flow using a CDC Biofilm reactor for 48 hours, scanning electron microscopy was used to determine morphology and total viable counts were made. Through this work it has been shown that the surface modification significantly influenced the distribution and morphology of the attached E. coli cells. What is more, it has been evidenced that the laser-modified PET has been shown to prevent E. coli cells from attaching themselves within the laser-induced micro-surface-features.

Keywords: KrF Laser, microbiology, E. coli, surface treatment, surface roughness

Introduction

In nature, microorganisms do not exist as planktonic (free-cell) individuals but predominantly exist by attaching to and growing as communities on living and inanimate surfaces. Such structures are known as biofilms, defined by Costerton *et al*. [1] as 'bacterial populations encapsulated within a matrix of secreted exopolymeric substances that are attached to a surface adherent to each other and/or surfaces or interfaces'. According to reports by a team of researchers at the Centre for biofilm Research in Montana [2], it was not until the 1970s that scientists began to appreciate that bacteria predominately exists as biofilms. Before this, bacteria was perceived as a unicellular life form existing in a pure-culture paradigm for the results to be experimentally valid, when in fact biofilm associated microbes are very different from their planktonic counterpart on a surface, displaying an altered phenotype in terms of growth rates and gene transcription, when compared to planktonic cells of the same organism [2-3]. It was not until the 1980s, early 1990s that scientists truly began to appreciate that biofilms are complex, well-organized and sophisticated communities and that the bacterial biofilm community is important [4].This has resulted in an inherent error in that this is not a true representation of how the majority of bacteria exist outside the laboratory environment [5].

Bacterial biofilms present major health concerns and economic burden in both hospital and industrial environments due to their ability to attach to various surfaces and the virulence associated with the biofilm mode of growth [6-14]. It has been estimated that hospital acquired infections cost the NHS up to £1000 million per annum [15]. Microbial activity and biofilms are also well known to cost the UK industry billions of pounds each year due to product contamination, energy losses and equipment damage.

The study of biofilms has grown markedly in recent years due to increased awareness of the pervasiveness and impact of biofilms on natural, human health and industrial systems. A novel area of research is the development of anti-biofouling surfaces. Such surfaces, which inhibit biofouling, are based on the modification to the physical surface structure of the substrate [16].

It is known that the physical properties of a surface regulates cell attachment and physiology. Surfaces which prevent this bacterial fouling through their physical structure represent a key area of research for the development of antibacterial surfaces for many different environments. Recent inspirations from nature have resulted in a range of novel surfaces possessing antimicrobial properties.

In a review by Donlan [17], he concluded that in general, although there are exceptions, the rougher and more hydrophobic the substratum is, the more rapidly a biofilm will form. This statement is further supported by Hsu *et al.* [16]; they investigated the effect of feature size of surfaces and periodicity on bacterial attachment. The group found that micro- and nanoscale topography does influence bacterial attachment, although there was no universal relationship between the surface topography and bacterial cell. Interestingly the group observed that cells seem to try and maximise contact area with surfaces resulting in specific alignment of cells depending on the arrangement of topographical detail. However, conflicting results have been reported in the literature, with some researchers finding a greater level of attachment to nanophase surfaces than to conventional surfaces, whilst others found a repellent effect of nanophase materials to bacterial cells [18].

Superhydrophobicity has been shown to prevent bacterial attachment. Nature uses rough surfaces on some plant leaves to produce a self-cleaning surface, also known as the lotus effect. The lotus effect is a superhydrophobic surface, meaning if a droplet of water was to be placed on the surface, contact angles would be measured of 150º or greater, enabling these droplets to roll and spin across the surface at very low tip angles, ≤5º [19 – 22]. Based on this, self-cleaning superhydrophobic products are now being engineered today. These products include: coatings that reduce the water drag on boats, building materials that remain clean with little maintenance, i.e. Pilkington Glass™, and textiles that are resistant to staining or remain dry when submerged in water [19, 23].

Laser surface treatment is a subject of considerable interest at present due to its ability to produce enhanced components with idealised surfaces and bulk properties [24]. Laser surface modification of polymeric materials has been extensively researched over the past decade resulting in wide application throughout different industries [25 – 35]. However, limited research has been focused on the surface modification of polymers for the prevention of bacterial attachment [18].

The present study investigates the relationship between laser-modified surface parameters, wettability characteristics and bacterial cell growth to develop a surface engineered technique to prevent or reduce the attachment of bacteria to surfaces relevant to the food and medical industries.

Materials and Methods

Laser surface modification

The Polyethylene terephthalate (PET) was sourced as coupons with 10 mm diameter and 3 mm thickness (BioSurface technologies, USA). The laser surface treatment of the coupons was commissioned from Micronanics Laser Solutions Centre, Oxford using Excimer laser with a wavelength of 248nm. Laser engineered pits measured 15µm in diameter with 20 µm wide gap between each pit. The patterns were produced using a projection etch technique with a repetition rate of 75Hz. The fluence at the work piece was calculated to be 0.58J/cm^2. 24 samples were etched in total.

Topography

Surface topography was analysed using a CCI profilometer (Micromearsure2, STIL, France). Sample sizes of 0.5mm x 0.5mm were examined for each of the coupon analysed. Three laser surface modified and three as-received control coupons were analysed. The results were analysed using SurfaceMaps (developed by STIL) and were expressed as R_a (the arithmetic mean of the departures of the roughness profile from the mean line) and S_a (the surface roughness calculated over an area) [36].

Surface contact angle

Sessile drop contact angles were analysed using a goniometer (OCA20; Dataphysics Instruments, GmbH). Prior to contact angle measurements being taken, samples were ultrasonically cleaned in acetone, ethanol then dH_2O for 3min each at room temperature (FB 11021, Fisher Scientific Ltd., UK). To ensure that the sample surfaces were dry, the coupons were placed in a specimen dryer overnight (LEEC, UK) before contact angle measurements were taken.

CDC Biofilm reactor

E. coli wild type ATCC strain 25922 was purchased as a 'cultiloop' from Oxoid Ltd, UK. Cultiloops were

stored at 5ºC until they were required. *E. coli* ATCC 25922 biofilm growth was analysed under high shear forces using a CDC Biofilm Reactor (CBR 90-Int, BioSurface Tec., USA).

Coupons were soaked in 10% (v/v) bleach solution for 15 minutes then rinsed twice with dH_2O and soaked in Teepol detergent (Teepol multipurpose detergent, Supply Trade Ltd., UK) overnight. Coupons were then rinsed twice with tap water and finally rinsed with distilled water.

Before the coupons were positioned and autoclaved the CDC biofilm reactor, Dust-OFF® (DPS, Dust-OFF®, USA) was used to gently blow any debris off the surface of the coupon. 500 ml of tryptone soy broth was added to the reactor beaker, the whole unit was then autoclaved on a standard sterilisation program, at 125ºC for 15 minutes.

An overnight culture of *E. coli* was diluted to an O.D. reading of 0.01 [37] before being added to the reactor which was set to run at 37ºC with an RPM to 125 for 2 days. Coupons were then aseptically removed and rinsed twice with sterile PBS to remove any planktonic bacteria before further analysis.

Viable Bacterial Counts

In order to enumerate the quantity of bacteria attached to the different coupons, each coupon was placed separately into a sterile 25 ml plastic universal tube containing 5ml PBS. The universals were then vortexed for 5mins, log diluted using PBS, and streak plated on LB agar plates. Plates were then incubated at 37ºC for 24 hours before analysing using an automated plate reader (Scan® 500, Interscience, France). Three laser modified and three as-received coupons were analysed, see table 1.

Scanning Electron Microscopy

Coupons were then prepared for SEM examination by washing with 0.1M sodium cacodylate and fixing in 2.5% glutaraldehyde in 0.1M sodium cacodylate for 30 minutes. Fixed specimens were then wash twice in dH_2O, dehydrated for 10 minutes at each stage of an ascending ethanol series (50% to 100%) and left to air dry (30). Each sample was coated in Au/Pd before being analysed under a FEI scanning electron microscope at a working distance of 10 mm (Quanta SEM, FEI™, USA).

Confocal Scanning Laser Microscopy

The preparation of Syto-9 was made based on the literature by Harrison *et al.* [38]. Working concentrations of 6.7 µM were prepared. Coupons to be analysed were aseptically placed with the side to be analysed facing upwards in a 24-well tissue culture plate. Ensuring that exposure to light was at a minimum, 1 ml of the prepared working concentration Syto-9 stain was dispensed carefully in to each well. The plate was then wrapped in tin foil, for protection from the light, and incubated static at 30ºC for 30 minutes. Afterwards, the stain was then gently rinsed off using 0.9% NaCl saline.

Coupons were visualised immediately using a Leica TCS Confocal microscope (TCS SP8, Leica Microsystems, UK).

Statistical Analysis

Comparison of the average total viable counts of the different coupon surfaces tested was performed using the t-test, two-sample assuming equal variances ($P = <0.05$). This statistical test assumed that the population variances were equal since the samples variances were almost the same. Comparison of the contact angle data for the different coupon surfaces tested was performed using a paired t-test ($P = <0.05$). Analysis was performed using Microsoft Excel 2013.

Results and Discussion

Surface Characterisation

Excimer laser surface modification was shown in this study to effectively irradiate the PET material to produce a regular symmetrical pattern on the surface see Figure 1. SEM analysis of the samples showed the surface of the laser patterned PET samples had been vastly modified in comparison to their as-received control sample.

The projection etch resulted in a symmetrical pattern of ‘pits’ evenly spaced across the coupon surface. The feature size of the laser drilled pits measured 15 µm in diameter with 20 µm spacing between each pit. Micronanics reported a pit depth of ~10µm.

On the laser treated coupons it is worth noting that it was still possible to see the surface features of the as-received untreated coupons, Micronanics also reported

quite an uneven surface when they laser treated the coupons. The effect of the laser treatment on the PET coupons produced a scale like gross appearance to the top surface of the coupon which visually appear to increase the surface roughness of the coupons.

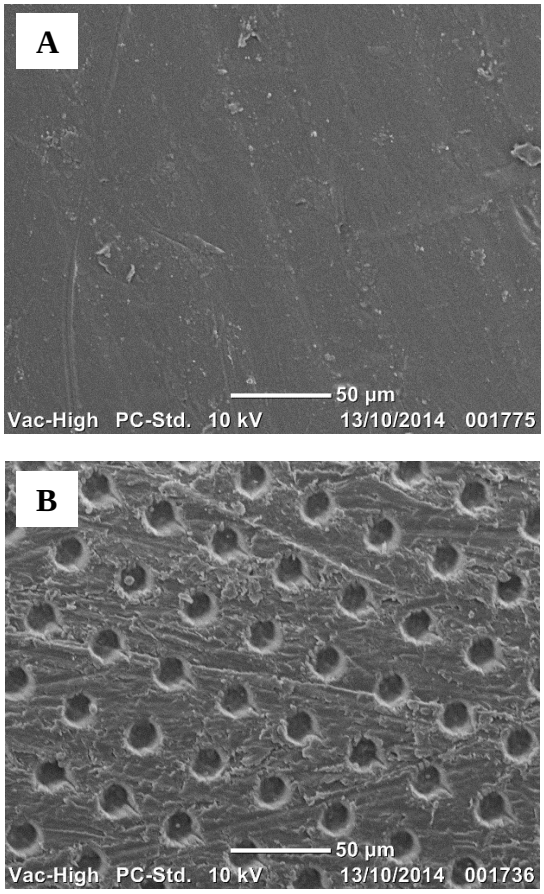

Figure 1: SEM images of (A) the as-received control sample and (B) the laser surface treated PET coupons

In order to obtain quantitative measurements of roughness parameters, the Micromeasure 2, 3D profiler was used. The R_a (the arithmetic mean of the departures of the roughness profile from the mean line) parameter was found to have considerably increased from 0.81±0.1 µm, up to 30.1±1.7 µm for the laser-induced patterned samples, see Table 1. The P-value was calculated to be <0.05, therefore the null hypothesis can be rejected and it was established that the laser surface treatment changed the wettability of the PET coupons, in this case increasing the hydrophobicity of the sample surface (see Table 1).

The S_a (the surface roughness calculated over an area) parameter was also shown to increase from the as-received value of 0.63±0.13 µm up to 42.9±7.80 µm. These high parameter values can be attributed to large crater-like surface features created by the Excimer projection etch as seen in Figure 1 and Figure 2.

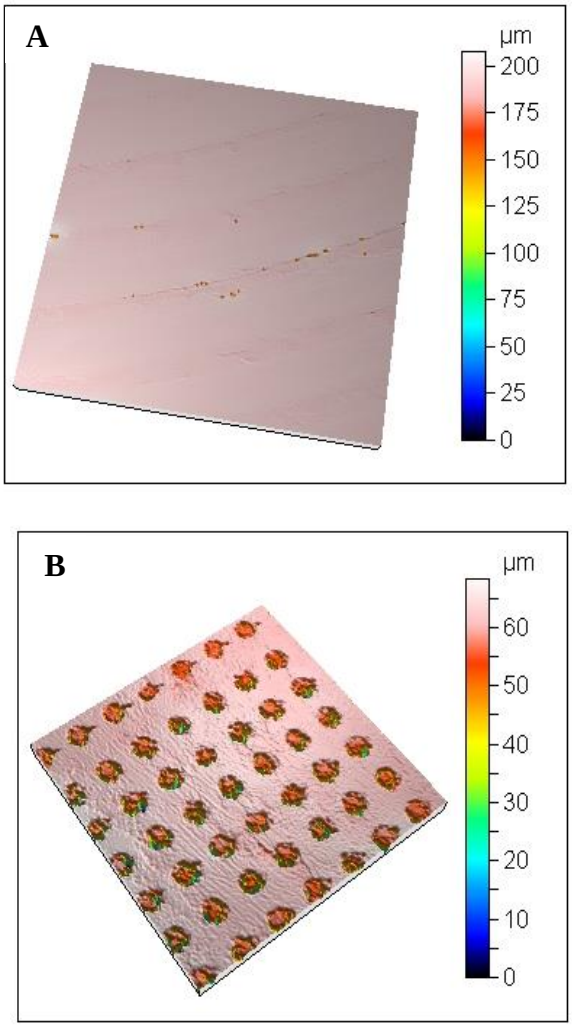

Figure 2: 3d profile image of the as-received control sample (A) compared to laser surface modified sample (B).

The R_a parameter value for all of the laser modified samples analysed was higher than the measured S_a value which can be explained due to the fact that the calculations derive from a single line whereas the Sa values takes in to consideration the complete surface therefore more realistic in determining the surface roughness. Again this may also explain why the R_a value for the as-received control sample differed.

Table 1: Summary of the average surface parameter and contact angle results for laser irradiated samples compared to the as-received control samples.

Sample	Sa (µm)	Ra (µm)	Contact Angle (°)
Laser patterned	42.9±7.80	30.1±1.7	87.69±5.34
As received	0.63±0.13	0.81±0.1	76.91±4.21

Regardless of this, the overall trend shows a large increase in surface roughness, which was expected due to the presences of the large crater-like pits.

Total Viable Counts

The total viable counts obtained from the attached bacterial cells to two different cell surfaces are presented in Table 2.

Table 2: Summary of total viable counts

Sample N°	Count	Dilution	CFU/mL
AGC01	37	1.00E-04	4.20E+06
AGC02	125	1.00E-03	1.44E+06
AGC03	24	1.00E-04	3.27E+06
	--- Average ---		**2.97E+06**
AGLS01	179	1.00E-03	2.14E+06
AGLS02	141	1.00E-02	1.53E+05
AGLS03	35	1.00E-03	4.78E+05
	--- Average ---		**9.24E+05**

On average it appears that the laser surface treated coupons reduced the number of colony forming units by a factor of 10; however, when the data was analysed using the student's t-test, two-samples assuming equal variances it was determined as not significant, P>0.05(see Table 2). Therefore it was not possible to determine if the different surface treatments, in comparison to the as-received control PET coupon, significantly reduced the number of attached bacterial cells to the coupon surface. With this in mind, more future experimentation is needed to confirm this.

Confocal Laser Scanning Microscopy

Visual observations of the confocal images (see Figure 3) show that attachment of bacteria to the surface of the laser treated coupons is localised to the surface surrounding the engineered pits. Comparing the preliminary data here to published work recently carried out investigating the influence of topography on the influence of bacterial attachment, there were major conflicts with the reported data [16, 43].

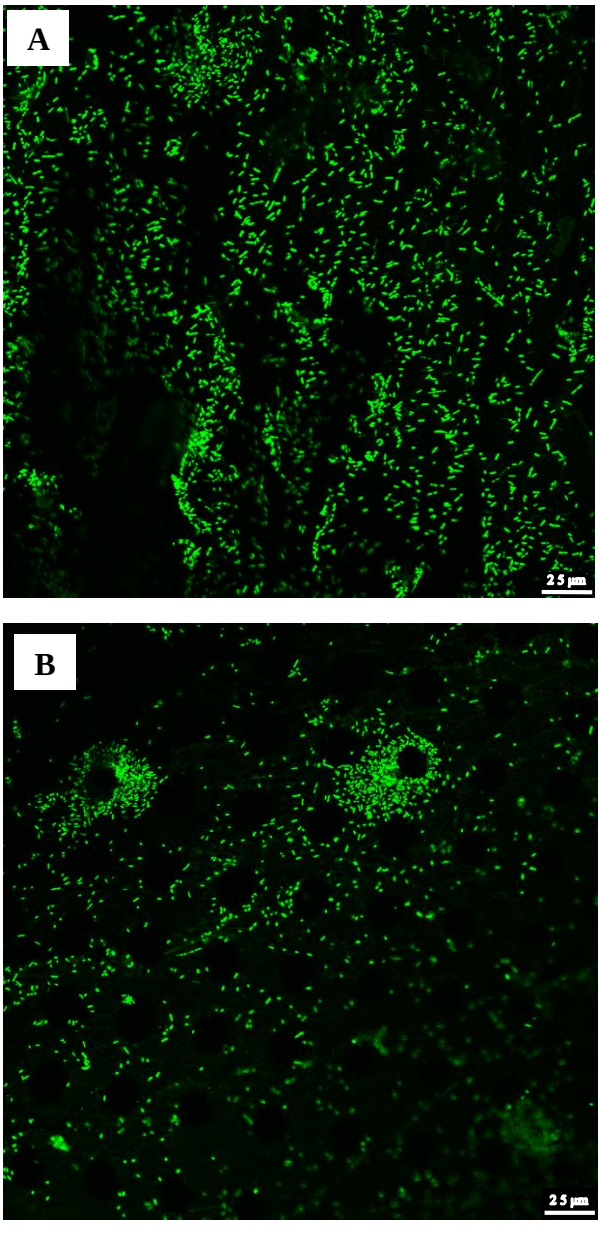

Figure 3: Confocal images of *E. coli* ATCC 25922 grown on polyethylene terephthalate (PET) after 48

hours. (A) Control surface PET coupons (B) laser surface treated PET coupons (x40 objective).

Vasudevan *et al*, [37] found that cells preferably attach to recessed features which contradicts the results presented in this study as the cells were found to be attached to the top surface surrounding the feature pits. However these observations were made on bacterial samples grown in static conditions, suggesting that shear forces play an influential role in the attachment of bacteria. The surface processing used by the workers to create the different topographical features was also different. The Vasudevan group [37] used standard photolithography process to prepare the PDMS patterned surfaces, therefore the influence of different surface chemistry must be important and taken in to consideration.

The confocal images of the laser modified pits (see Figure 3) show that, in places, the bacterial load was concentrated around the engineered pits. Furthermore, Figure 3 shows that within the pits, there was no presence of bacterial cells. Previous studies have observed that cells try and maximise the contact area with surfaces resulting in specific alignment of cells depending on the arrangement of topographical detail [16]. This observation could also be used in this instance to explain the preference for the bacterial cells to adhere to the flat surface of the control (as-received) surface, rather than the edges of the laser engineered pits, which could have potentially reduced the surface area available for the cells to adhere to.

It is also known that superhydrophic surfaces, surfaces which have contact angles exceeding 150^{o}, have been shown to repel bacterial attachment [42]. In this study, both the contact angle measurements and the surface parameter measurements of the laser surface modified coupons increased compared to the values of the as-received control sample which, based on the literature would suggest an expectation for a higher bacterial load on the laser modified coupons. However, for many pathogens, the interaction between bacterial cell and potential host surface determines the ability of the microorganism to colonise and infect the host [16]. As such, the solid surfaces which were developed using laser surface engineering may have had many different parameters which could be influencing the attachment of the *E. coli* in this study. On account, of this further research is required to explore these other potential parameters such as surface charge, functional groups and nutrient availability [16, 43].

The total viable count results show that the bacterial load on the laser modified coupons was approximately 10 fold lower than that of the as-received control coupons. Although the count difference was found not to be significant, this was an unexpected result as the significant increase in hydrophobicity as determined by the roughness and contact angle results suggested the load of bacteria should in fact be greater. As a result, it has been evidenced with this work that laser engineering of the topography at a microscale level does have an influence on the attachment of *E. coli* to laser modified surfaces.

It is known that the presence of patterning leads to the incorporation of air pockets on immersion of the surface in to liquid [37]. In the present study, it is possible that a water-air-solid interface formed due to the specific patterning of the surface when the coupons were immersed in the liquid broth and could explain why the bacterial cells were unable to attach within the engineered pits. This stable state where a droplet is suspended over an air pocked without complete interfacial adhesion of the liquid to the solid surface is defined as the Cassie-Baxter state [37, 40, 41]. Vasudevan *et al.* [37] discusses the importance of having a stable state composite of water-air-solid interface of a patterned surface to prevent bacterial fouling by promoting stable state air pockets, produced by surface patterning. It is possible that this phenomenon took place on the laser engineered samples and gave rise to what was observed in Figure 3b showing bacterial cells attached to the surface of the coupon surrounding the laser engineered pits.

Interestingly, in our study, the laser surface treated surfaces showed more features of a mature biofilm structure [17], whereas the non-laser surface treated coupons displayed less signs of a mature biofilm, which was apparent on all the laser modified samples tested. It may be possible to conclude that the laser treated coupons facilitate or promote the growth of mature biofilm in comparison to the control PET surface for reasons we do not as yet understand. Further analysis would be required to investigate this including statistical comparison of bacterial coverage.

Conclusions

Excimer laser surface modification was shown in this study to effectively irradiate the PET material to produce projection etch pattern of the materials surface. Laser surface treatment of the PET coupons influenced the attachment of bacteria, notably inside the feature pits. However, laser coupons may have promoted maturity in biofilm growth. The hydrophobicity of the laser surface modified coupons was significantly increased and the distribution of the attached *E.* coli

cells changed from being scattered across the surface of the coupon to localising around the engineered pits. It should also be noted although the laser surface treatment of the PET coupon did not significantly ($P>0.05$) reduce the number of bacterial cells attached to the coupon surface that there was an overall reduction in numbers.

Due to the change in bacterial attachment to the laser surface modified coupons, it is possible to conclude that that laser engineering of the topography at a microscale level does have an influence and, through further research and development, could provide a potential methods for the prevention of the attachment of *E. coli* to modified surfaces.

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